UNIVERSITI PUTRA MALAYSIA

CALLUS FORMATION, SOMATIC EMBRYOGENESIS AND DEVELOPMENT OF TRANSFORMATION PROTOCOL BY PARTICLE BOMBARDMENT IN PLANTAIN BANANA

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CALLUS FORMATION, SOMATIC EMBRYOGENESIS AND DEVELOPMENT OF TRANSFORMATION PROTOCOL BY PARTICLE BOMBARDMENT IN PLANTAIN BANANA

By

PUSPITA DESWINA

Thesis Submitted in Fulfilment of the Requirement for the Degree of Master of Science in the Faculty of Science and Environmental Studies

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Dedicated To:

My Parents: Papa Yuzar Akmam and Mama Djawanis

My beloved husband: Heroriki
My dearest son: Ibnu Khalil Ibram
for their constant love during my study

Brothers and Sister: Dodi Indra (Uda Dodi); Media Sandra Kasih (Uni Med); Jecky Aulia(Tet)

Abstract of thesis presented to senate of Universiti Putra Malaysia in fulfilment of requirement for the degree of Master of Science

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Chairman : Professor Hjh. Marziah Mahmood, Ph.D.

Faculty : Science and Environmental Studies

The plantain bananas are among the most valued crop plants in the tropical world. However, the commercially attainable yields are very low compared to dessert bananas. Conventional breeding of this plant remains a difficult endeavor because of high sterility and polyploidy; therefore, the integration of biotechnology into plantain improvement programmes is essential. Somatic embryogenesis has the great potential for rapid and efficient regeneration of plantlets and offers opportunities for large-scale production of plant material. The yield and quality of somatic embryos produced in cell culture depends on the media constituents and explants type. In this study, *in vitro* rhizomes initiated from shoot tips (8 week-old cultures) of cultivars Nangka and Tanduk and immature male flowers of cultivar Nangka were used as explants. The results of callus initiation showed that treatments with 2,4-D, Picloram and Dicamba produced callus ranging from 0.0–82.0% for cultivar

Nangka and 0.0–58.0% for cultivar Tanduk. The type of callus varied depending on concentrations of PGRs used for the initiation. However, no callus formation was obtained from the auxin-free medium. The study on the determination of callus growth curve showed that treatments with 2,4-D and Picloram for both cultivars Nangka and Tanduk produced a sigmoidal pattern, except for several concentrations which failed to show the callus growth pattern. Whereas, for the Dicamba treatments, there was no callus growth at all. The effects of two basal media supplemented with 2,4-D and Picloram at different concentrations were studied. The highest callus fresh weight (0.37 g) was attained on SH medium plus 2.5 μM Picloram. The effect of 2,4-D and Picloram in combination with other auxins and cytokinins, showed that there was no constant trend on callus growth in response to the treatments. However, the treatments using 2,4-D and Picloram in combination with other cytokinins produced nodular and compact callus. The callus produced embryogenic structure (13.3–66.7%) in the somatic embryogenesis medium, but no shoot regeneration was achieved, instead only roots were formed. In the initiation of callus from immature male flowers, 49.7% of flower cluster responded to form globular embryogenic callus and the highest response was from flower of rows 12 and 13 (60%). The study on the development of transformation protocol by particle bombardment using *in vitro* rhizome slices and embryogenic callus as explants and GFP fluorescent gene as the reporter gene revealed an unstable transient expression in the cell.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master of Sains

PEMBENTUKAN KALUS, EMBRIOGENESIS SOMA DAN PENGHASILAN PROTOKOL TRANSFORMASI MELALUI PEMBEDILAN ZARAH DI DALAM PISANG PLANTAIN

Oleh

PUSPITA DESWINA

Disember 2001

Pengerusi : Profesor Hjh. Marziah Mahmood, Ph.D.

Fakulti : Sains dan Pengajian Alam Sekitar

Pisang plantain adalah diantara kebanyakan tanaman yang bernilai tinggi di kawasan tropika. Walau bagaimanapun, hasil keluaran komersil yang diperolehi daripadanya sangat rendah berbanding dengan pisang pencuci mulut. Pembiakan biasa tanaman ini masih sukar disebabkan oleh kadar kesterilan dan poliploidi yang tinggi. Oleh itu, integrasi bioteknologi dalam program memperbaiki pisang plantain memerlukan protokol kultur sel yang sesuai, di mana embriogenesis soma mempunyai potensi yang tinggi untuk pembiakan dalam jumlah besar, cepat dan cekap. Hasil dan kualiti daripada embrio soma yang dihasilkan dalam kultur sel bergantung kepada kandungan media dan eksplan berbeza yang digunakan dalam proses pengkulturan tersebut. Dalam kajian ini, dua jenis eksplan telah diguna iaitu rizom *in vitro* daripada kultivar Nangka dan Tanduk serta bunga jantan yang belum matang daripada kultivar Nangka. Keputusan yang diperolehi daripada inisiasi kalus menunjukkan rawatan dengan 2,4-D, Picloram dan Dicamba menghasilkan pembentukan

kalus antara 0.0–82.0% daripada kultivar Nangka dan 0.0–58.0% untuk kultivar Tanduk. Jenis kalus berbeza-beza bergantung kepada kepekatan pengawalatur tumbesaran yang digunakan untuk inisiasi. Walau bagaimanapun, tidak terdapat pembentukan kalus daripada media tanpa auksin. Kajian keatas penentuan pertumbuhan kalus dengan rawatan 2,4-D dan Picloram untuk kultivar Nangka dan Tanduk telah dapat menghasilkan lengkung pertumbuhan sigmoid, kecuali bagi beberapa kepekatan. Manakala rawatan dengan Dicamba, tidak menghasilkan pertumbuhan kalus. Kesan daripada dua media dasar yang dibekalkan dengan 2,4-D dan Picloram pada kepekatan yang berbeza menghasilkan gumpalan kalus yang banyak dalam media SH ditambah dengan 2.5 μM Picloram. Kesan 2,4-D dan Picloram dengan gabungan auksin dan sitokinin yang lain menunjukkan tidak terdapat kecenderungan yang tetap diperolehi diantara rawatan. Walau bagaimanapun, pada rawatan 2,4-D dan Picloram yang digabung dengan sitokinin yang lain boleh menghasilkan kalus yang bernodul dan padat, kalus ini bertukar kepada struktur embriogenik (13.3 – 66.7%) di dalam media embriogenesis soma, tetapi tidak ada regenerasi pucuk yang diperolehi. Walau bagaimanapun tindakbalas embriogenik hanya membentuk akar. Sebaliknya, inisiasi kalus daripada bunga jantan yang belum matang hanya menghasilkan kalus embriogenik bernodul (±49.7%) dan kelompok yang berpotensi membentuk kalus bernodul adalah pada baris 12 dan 13 (60%). Kajian terhadap pembangunan protokol transformasi melalui pembedilan zarah menggunakan potongan rizom *in vitro* dan kalus embriogenik sebagai eksplan, dan GFP gen berpendaflor sebagai gen penanda didapati pengutaraan transien ke dalam sel tidak stabil.

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May ALLAH bless us always.....

I certify that an Examination Committee met on 7th December 2001 to conduct the final examination of Puspita Deswina on her Master of Science thesis entitled "Callus Formation, Somatic Embryogenesis and Development of Transformation Protocol by Particle Bombardment in Plantain Banana" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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Date: 14 MAR 2002

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

PUSPITA DESWINA

Date : 2 JAN 2002

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%	Percentage
°C	Degree Celcius
2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-benzylaminopurine
br	Browning
cm	Centimetre
conc.	Concentration
ctrl	Control
cul.	Culture
d	Day
dH_2O	Distilled water
Dicamba or Dic	3,6-Dichloro-o-aniscic acid
EDTA	Ethylenediaminetetrraacetic acid (ferric sodium salt)
e.g.	Example
EtOH	Ethanol
FW	Fresh weight
g	Gram
h	Hours
i.e.	That is
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kinetin or Kin	6-furfurylaminopurine
L	Litre
mg	Milligram
mm	millimeter
min	Minute
mL	Millilitre
mM	Millimolar
Na2EDTA	EDTA disodium salt
NAA	α-Naphtaleneacetic acid
NaOH	Sodium hydroxide
NPT II	Neomycin phosphotransferase
nr	no response
nm	nanometre
PGR(s)	Plant growth regulator(s)
Picloram or Pic	4-Amino-3,5,6-trichloropicolinic acid
SDS	Sodium dodecyl sulphate
Zeatin	6-(4-Hydroxy-3-methyl-but-2-enylamino) purine
v/v	Volume for volume
w/v	Weight for volume
N	Normality
μ	Specific growth rate (d)
μl	Microlitre
μg	Microgram
μM	Micromolar

CHAPTER I

INTRODUCTION

1.1. Importance of Plantains

Plantains are important staple food crops for certain groups of people living in the tropics and they are among the cheapest sources of starch (Swennen, 1990; Rowe, 1998). According to Dadzie (1995) plantain banana has practically the same nutritional value as dessert banana, but the carbohydrate content mainly consists of starch rather than sugar. In general, plantain and banana provides a good source of carbohydrates, minerals such as potassium, magnesium, phosphorous, calcium, iron and also vitamins A and C (Horry, 1990; Kodym and Zapata-Arias, 1999). Latham (1979) reported that banana starch is easily digested and is thus suitable for the preparation of food for infants. On the other hand, the nature of the carbohydrates varies widely between cultivars and within a particular cultivar during different stages of ripening (Vuylsteke *et al.*, 1990).

Plantains consisted of one-half of the total world output of bananas (Swennen, 1990). Nevertheless, several countries make no distinction in their statistics between banana and plantain production and publish only the overall estimates (FAO, 1998). To date, very little progress has been made in the improvement of plantain banana; therefore, it is important for agriculturists to

develop a systematic research program in improving plantain banana as a food crop.

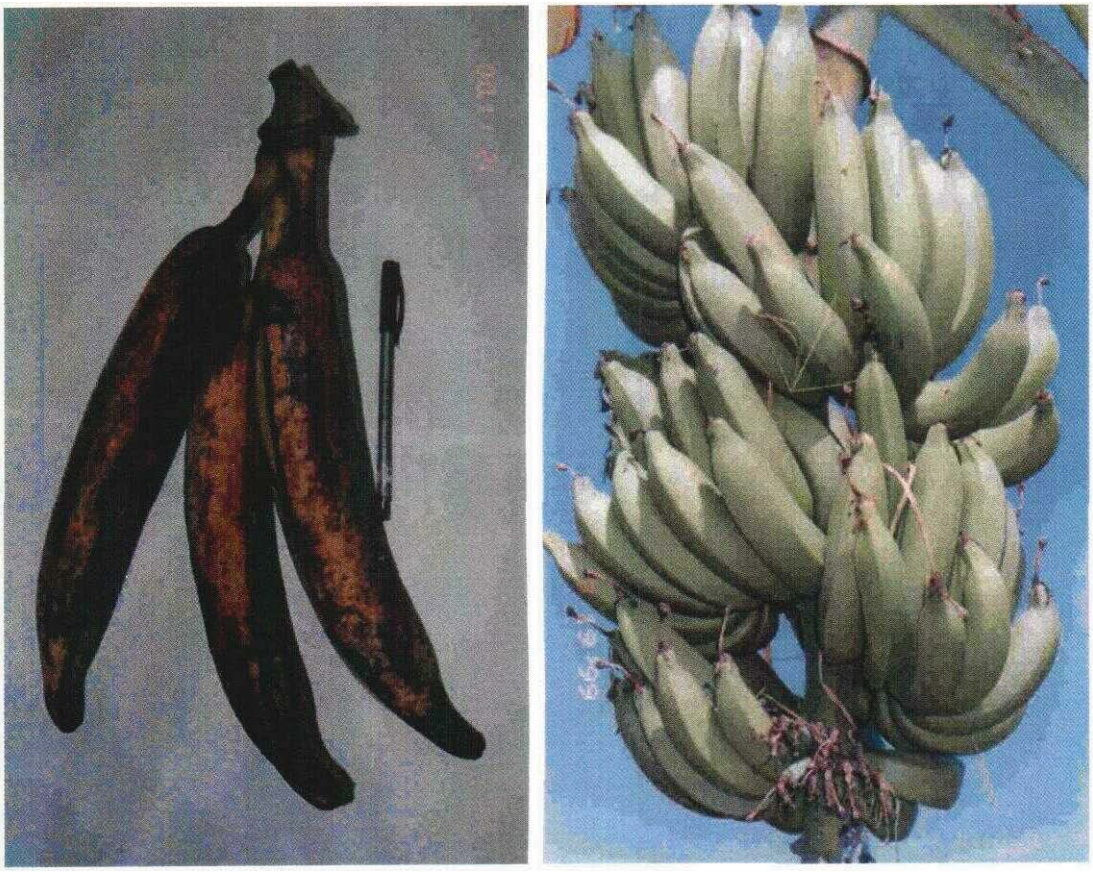
In this study, two plantain cultivars (Nangka and Tanduk) which are commercially popular in Malaysia (Jamaluddin, 1990) were investigated.

- Cultivar Nangka (AAB)

The fruit skin is thick and remains light green in color when ripe. The flesh is creamy white, fine textured, starchy and subacid in taste. The fruit ranges from 18 to 24 cm in length, 3.5 to 5.0 cm wide and has good keeping quality. The large hands have 14 to 24 fingers and the fruit bunch has 6 - 8 hands (Kusumo and Dasi, 1989) (Plate 1.1a).

- Cultivar Tanduk (AAB)

It is popular known as "horn plantain", and is also the largest fruit among the bananas. It has limited commercial value in Malaysia due to its poor yield. The fruit has excellent keeping quality and remains starchy even when fully ripe, and requires cooking to become palatable. The fruit ranges from 25.0 to 35.0 cm in length and from 5.0 - 6.5 cm in diameter. The bunch weight is only 7 - 10 kg. The skin is yellow in colour when ripe and the pulp is



(a) (b)

Plate 1.1 : *Musa* cultivars (a) Tanduk (b) Nangka

light colour with fine but firm texture. Generally, there are two hands in a bunch and sometimes only one hand. There are no male flower buds formed for this cultivar (Swennen, 1990) (Plate 1.1b).

1.2. Improvement of Plantains and Bananas through Biotechnology

Plantain and banana production is hampered by several diseases such as black Sigatoka , *Fusarium* wilt and banana bunchy top virus (BBTV) disease, which resulted in an increased effort to genetically improve the crop (Vuylsteke *et al.*, 1993; Lee *et al.*, 1997). Robinson (1996) reported two major problems in all plantain cultivars which are highly susceptible to the black Sigatoka fungus, and commercially low attainable yields compared to dessert bananas. Conventional breeding of *Musa* spp. poses problems such as high sterility and polyploidy nature of most of the edible cultivars (Vuylsteke *et al.*, 1993; May *et al.*, 1995; Schoofs *et al.*, 1997). Therefore, biotechnological approaches such as through mutation breeding, somaclonal variation or genetic engineering may have great potential in the genetic enhancement of plantains and bananas. Hence, some scientists advocated these biotechnological approaches as the solution to genetically improve the crop.

Crouch *et al.* (1998) stated that genetic modification of *Musa* spp. offers the opportunity to add desirable gene characteristics into target tissue by genetic transformation technique. The establishment of a transformation protocol is necessary which includes the preparation of a target material for the

transformation and the regeneration of transformed plant. One of the target materials used in the transformation of *Musa* spp. is somatic embryo. Somatic embryos have proven to be the ideal materials as plantlets produced are non-chimeric and the multiplication is rapid (Panis and Swennen, 1993). The potential use of somatic embryogenesis for rapid and efficient regeneration of plantlets is practically essential in genetic transformation work (Crouch *et al.*, 1998 and JayaSree *et al.*, 2001). Moreover, somatic embryogenesis is useful in agriculture because it fixes the genotype to that of the female parent (Grapin *et al.*, 2000).

The genetic improvement of this crop through the transfer of foreign genes into target cells have been achieved using embryogenic cell suspension (Panis and Swennen, 1993; Sagi *et al.*, 1995;
al., 2001), protoplasts cell (Sagi *et al.*, 1994; 1995) and rhizome slices (May *et al.*, 1995) by *Agrobacterium*-mediated transformation and particle bombardment. However, the regeneration of whole plants from cultured somatic cells is still a major limiting step in the application of genetic engineering for the improvement of banana crop (Novak, 1992; Crouch *et al.*, 1998; Kodym & Zapata-Arias, 1999) and success is also genotype-specific (Rout *et al.*, 2000).

This project is divided into two parts, the induction of callus and somatic embryogenesis, and the development of transformation protocol in cultivars Nangka and Tanduk.